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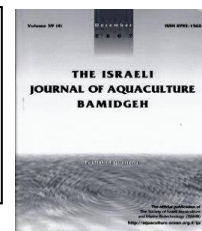
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Anesthetic Efficacy of *Cymbopogon citratus* Essential Oil as a Herbal Agent in Two Ornamental Fish Species

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Key words: Anesthesia; *Cymbopogon citratus*; *Sciaenochromis fryeri*; *Labidochromis caeruleus*.

Abstract

The efficacy of *Cymbopogon citratus* essential oil (CcEO) as a herbal anesthetic agent was evaluated for two ornamental fish species, *Sciaenochromis fryeri* and *Labidochromis caeruleus*. Fish were exposed to various essential oil concentrations and the lowest effective concentrations were determined according to deep anesthesia ($A_D < 3$ min) and full recovery ($R_F < 10$ min) times. CcEO showed anesthetic traits and induced A_D in *S. fryeri* and *L. caeruleus*. The lowest effective concentrations was 200 µl/L for *S. fryeri* (A_D ; $R_F \rightarrow 122 \pm 1.8$ s; 638 ± 8.9 s) and 200 µl/L for *L. caeruleus* (A_D ; $R_F \rightarrow 139 \pm 6.9$ s; 625 ± 11.1 s). At concentration of 25 µl/L *C. citratus* essential oil did not induce A_D in both of ornamental fish species. No significant differences were found between fish species in terms of induction and recovery times for same concentrations excluding A_D time of 200 µl/L concentration. Although the fastest A_D were obtained by 300 µl/L, recovery times were prolonged and fish did not full recover in 20 min. Induction and recovery times for CcEO were significantly dependent on concentrations and negative relationships were recorded between A_D and R_F . Neither mortality nor adverse effects were observed on two fish species. As a result, CcEO showed anesthetic properties for *S. fryeri* and *L. caeruleus*.

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Introduction

Ornamental fish are commercially valuable for aquarium hobbyists and merchants. *Sciaenochromis fryeri* and *Labidochromis caeruleus* are popular worldwide in the aquarium industry. Lake Malawi is home to hundreds of cichlid species, most of which are endemic to the lake. *S. fryeri* are widespread in Lake Malawi and particularly abundant in intermediate areas of the lake (Gerlai, 2007). The bright blue coloration in males is the most prominent feature of *S. fryeri*. In this species, the males naturally attain a bright blue coloration at the onset of sexual maturity while females and young fry are silvery gray (Golan and Levavi-Sivan, 2014). *L. caeruleus* is a cichlid freshwater fish and has a wide depth range in Lake Malawi (Lewis, 1982). This species has bright or pale yellow coloration depending on their nutrition.

Some handling procedures, such as morphometric measurements, grading, or transporting of these fish, cause stress that influences fish behavior and physiology adversely (Ross and Ross, 2008). Immersion anesthetics are used in hatchery settings by veterinarians, field biologists, and laboratory researchers to aid in handling finfish for medical procedures and research purposes (Silbernagel and Yochem, 2016). To minimize the negative impact of handling on fish, anesthesia is an ideal option and carried out at this point for eliminating the adverse effects of stress.

Anesthetics such as MS-222, 2-phenoxyethanol and clove oil are commonly used in aquaculture. An ideal anesthetic agent should be harmless to user, fish, and the environment (Kizak et al., 2013). It has also been reported that several synthetic anesthetic agents have some side effects on fish and humans (Velisek et al., 2007; Zahl et al., 2012; Fernández-Parra et al., 2017). From this point, DIRECTIVE 2010/63/EU appears to be a crucial obligation for ensuring fish welfare. Alternative new herbal essential oils as natural anesthetics have become preferable to synthetic agents.

Many studies have been carried out on various herbal essential oils as anesthetic agents such as *Lippia alba*, *Hesperozygis ringens*, *Lippia sidoides*, *Ocotea acutifolia*, *Cinnamomum camphora*, *Mentha arvensis*, *Aloysia triphylla*, *Cymbopogon flexuosus*, *Matricaria chamomilla*, *Origanum* sp., *Eucalyptus* sp., *Melaleuca alternifolia*, *Pelargonium graveolens* and *Aniba rosaedora* (Cunha et al., 2010; Silva et al., 2013; Pedrazzani and Neto, 2016; Santos et al., 2017; Can et al., 2017; Bodur et al., 2018; Correia et al., 2018; Can et al., 2018; Kizak et al., 2018).

Cymbopogon citratus, commonly known as lemon grass, has been used for medicinal purposes for many years (Onawunmi et al., 1984) as a spasmolytic, analgesic, anti-inflammatory, antipyretic, diuretic and tranquilizer. Several clones have been cultivated for the production of the essential oil which is widely used in the cosmetic industry and for chemical synthesis, due to its high content of citral, a natural mixture of two isomeric aldehydes, neral and geranial (Viana et al., 2000).

To date, CcEO has not been used as an anesthetic agent in fish. This study aims to investigate the efficacy of CcEO as a novel and eco-friendly herbal anesthetic agent on two ornamental fish species.

Material and Methods

Experimental fish and water quality.

Trials were conducted on two ornamental fish species, *S. fryeri* (mean body weight 1.45 ± 0.23 g) and *L. caeruleus* (mean body weight 1.38 ± 0.19 g) (n=91). Both species were purchased from a private pet-shop in Elazig (Turkey) and were transferred to Laboratory of Bioengineering Department, Munzur University (Turkey). Fish were stocked into two glass aquaria (each one 200 L) and gradually acclimatized to water. They were fed ad libitum once daily at approximately 1.5% of their own weight with commercial granular feed (Sera Granured) containing 42.5% crude protein. They were starved 24 h prior to start of the trials. Water quality parameters were about 24.5°C, pH 7.8, and DO 7.0 mg/L during the experiment.

Anesthetic agent.

An essential oil was investigated as a herbal anesthetic agent in this study. CcEO was purchased from a commercial company (Botalife, Turkey). The essential oil components

were analyzed at the Ege University Center for Drug R & D and Pharmacokinetic Applications Environmental & Food Analysis Laboratories (ARGEFAR, Izmir, Turkey) by using GC-MSD instrument. The main components of CcEO are given in Table 1.

Table 1. Main components of CcEO.

| Component | Percentages (%) |
|---------------------------|-----------------|
| Geranial | 44.74 |
| Neral | 31.00 |
| Geraniol | 8.08 |
| Geranyl acetate | 5.98 |
| Trans Caryophyllene | 2.52 |
| Gamma Cadinen | 1.61 |
| 6-methyl – 5-hepten-2-one | 1.39 |
| Linalool | 1.10 |
| Caryophyllene oxide | 1.07 |
| Camphene | 0.72 |
| 4-Nonanone | 0.68 |
| Alpha cubebene | 0.53 |
| TOTAL | 99.42 |

CcEO was initially dissolved in ethanol because of its insolubility. Preparation of essential oil concentration was performed according to the method of Can et al. (2018) where the oil was poured into a 15 ml plastic capped tube at a desired amount and then 10 X ethanol (94% purity) was added to each tube. After shaking, 10 ml of treatment water was added into a solution of essential oil + ethanol solution and shaken again. Finally, the solution was poured into a glass aquarium filled with 1 L of treatment water.

Experimental design.

S. fryeri and *L. caeruleus* fish species were exposed to six concentrations of the CcEO (25, 50, 75, 100, 200 and 300 µl/L) to determine the optimal induction and recovery times. Determination of anesthesia (A) and recovery (R) stages are shown in Table 2.

Table 2. Anesthesia stages and description of fish behaviour (A: Anesthesia, R: Recovery) (Mylonas et al., 2005; Kizak et al., 2018).

| Stages of A and R | Description of Fish Behaviour |
|------------------------------------|---|
| A _I (Initial Induction) | Total loss of equilibrium, slow but regular opercular rate |
| A _D (Deep Anesthesia) | No reflex, opercular movements slow and irregular, no respond to strong external stimulus |
| R _I (Initial Recovery) | Total recovery of equilibrium, swimming erratic |
| R _F (Full Recovery) | Total behavioural recovery, normal swimming |

Each fish was assessed individually and used only once in each replicate at defined concentrations. Seven fish were tested to find the lowest effective concentration that was chosen according to following criteria: (A_D) should be achieved in less than 3 min and the full recovery (R_F) time should not exceed 10 min. Induction and recovery times of different concentrations for essential oil were recorded with a digital stopwatch. Fish were taken from the holding tank in a net, transferred individually into a glass aquarium (1 L) and observed for equilibrium, opercular movements and response to tail pinch. When they lost total equilibrium and reached A_D stage, the fish were removed by net, weighed and transferred to anesthesia-free water to assess recovery time. After R_F stage, fish were transferred into a holding tank, and monitored for survival and abnormal behaviour for 24 hours. Additionally, a solution with 3 ml/L of ethanol was tested in seven fish within maximum exposure time (3 min) to examine the efficacy of ethanol and determine whether it had an anesthetic effect or not on fish.

Statistical analysis.

Normality and homogeneity of data were checked to comply with the assumptions of ANOVA. One-way ANOVA followed by the Duncan Multiple Range test was used to determine significant differences among means. Results are presented as means ± SE and statistical significant differences are expressed as $p < 0.05$. All statistical analyses were carried out using SPSS (Version 20.00). Regression equations, performed with Excel, showed relationship between anesthetic concentrations and induction/recovery times as well as between induction and recovery times.

Results

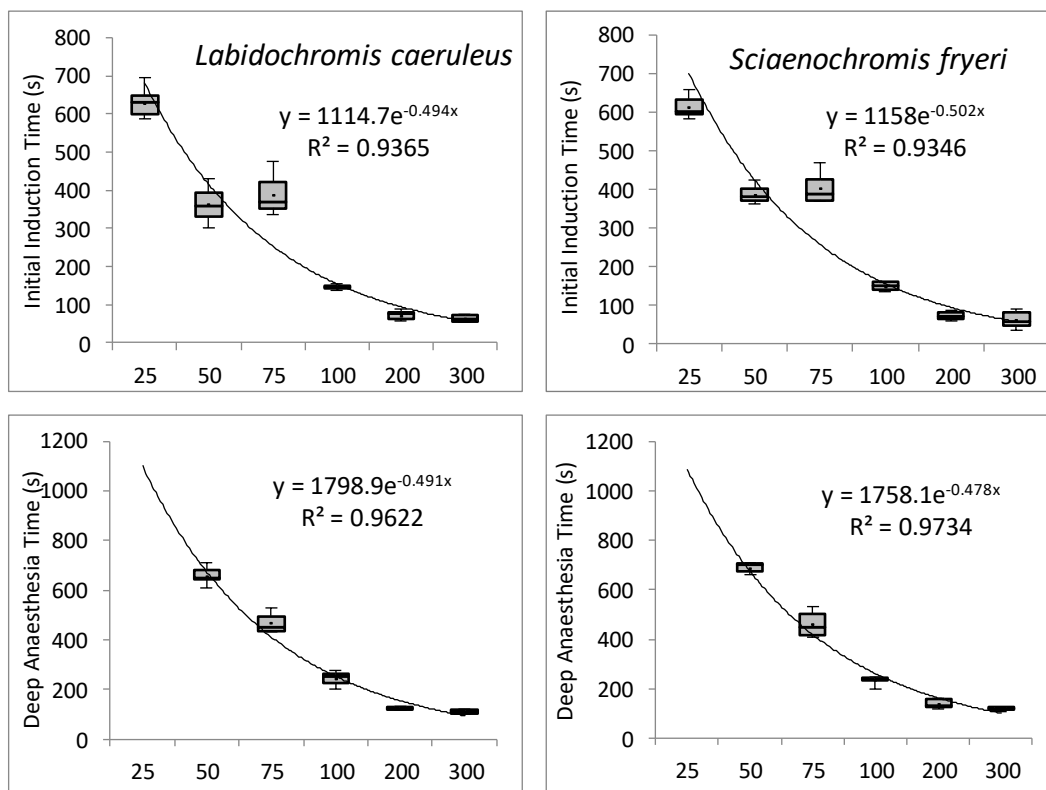
CcEO showed anesthetic characteristics, and induction was achieved with most of the applied concentrations on *S. fryeri* and *L. caeruleus*. No mortality or adverse effects were observed during the treatments. Induction and recovery times with statistical differences ($p < 0.05$) are shown in Table 3.

Table 3. The anesthesia stage times at different concentrations of CcEO for *Sciaenochromis fryeri* and *Labidochromis caeruleus*.

| <i>Sciaenochromis fryeri</i> | | | | |
|----------------------------------|---------------------|------------------|-------------------|------------------|
| Concentration $\mu\text{L/L}$ | Induction Time (s) | | Recovery Time (s) | |
| | A_I | A_D | R_I | R_F |
| 25 | 615 ± 10.5^g | n.o. | n.t. | n.t. |
| 50 | 388 ± 8.3^{ef} | 658 ± 13.8^g | 113 ± 4.5^a | 254 ± 11.7^a |
| 75 | 403 ± 16.2^f | 467 ± 14.6^f | 178 ± 6.1^d | 393 ± 6.1^c |
| 100 | 149 ± 4.0^d | 243 ± 11.1^e | 166 ± 4.0^c | 512 ± 6.6^d |
| 200 | 71 ± 3.7^{bc} | 122 ± 1.8^c | 334 ± 1.7^f | 638 ± 8.9^e |
| 300 | 61 ± 8.2^{ac} | 110 ± 3.0^a | 15 min up | 21 min up |
| <i>Labidochromis caeruleus</i> | | | | |
| Concentration $\mu\text{L/L}$ | Induction Time (s) | | Recovery Time (s) | |
| | A_I | A_D | R_I | R_F |
| 25 | 630 ± 14.4^g | n.o. | n.t. | n.t. |
| 50 | 362 ± 18.2^e | 690 ± 7.6^g | 106 ± 6.3^a | 285 ± 8.1^b |
| 75 | 389 ± 21.1^{ef} | 460 ± 19.1^f | 151 ± 2.9^b | 380 ± 8.9^c |
| 100 | 145 ± 2.3^d | 233 ± 6.0^e | 166 ± 5.9^c | 513 ± 6.4^d |
| 200 | 72 ± 4.3^{bc} | 139 ± 6.9^d | 326 ± 3.1^e | 625 ± 11.1^e |
| 300 | 63 ± 3.6^a | 115 ± 1.8^b | 15 min up | 21 min up |

Treated fish at 50, 75, 100, 200 and 300 $\mu\text{L/L}$ concentrations of CcEO reached deep anesthesia (A_D), while the concentration of 25 $\mu\text{L/L}$ did not induce stage A_D for both fish species. The lowest effective concentrations for CcEO were established at 200 $\mu\text{L/L}$ for both fish

species. No significant differences were found between fish species in terms of induction and recovery times for same concentrations ($p > 0.05$) excluding A_D time of 200 $\mu\text{L/L}$ concentration ($p < 0.05$). Anesthesia times were decreased with increasing concentrations, while recovery times increased (Fig. 1). Even though the fastest A_D was obtained by 300 $\mu\text{L/L}$ concentrations for both fish species, recovery times (R_I and R_F) did not occur in within 15 min. All the treated fish recovered fully after 21-22 min.



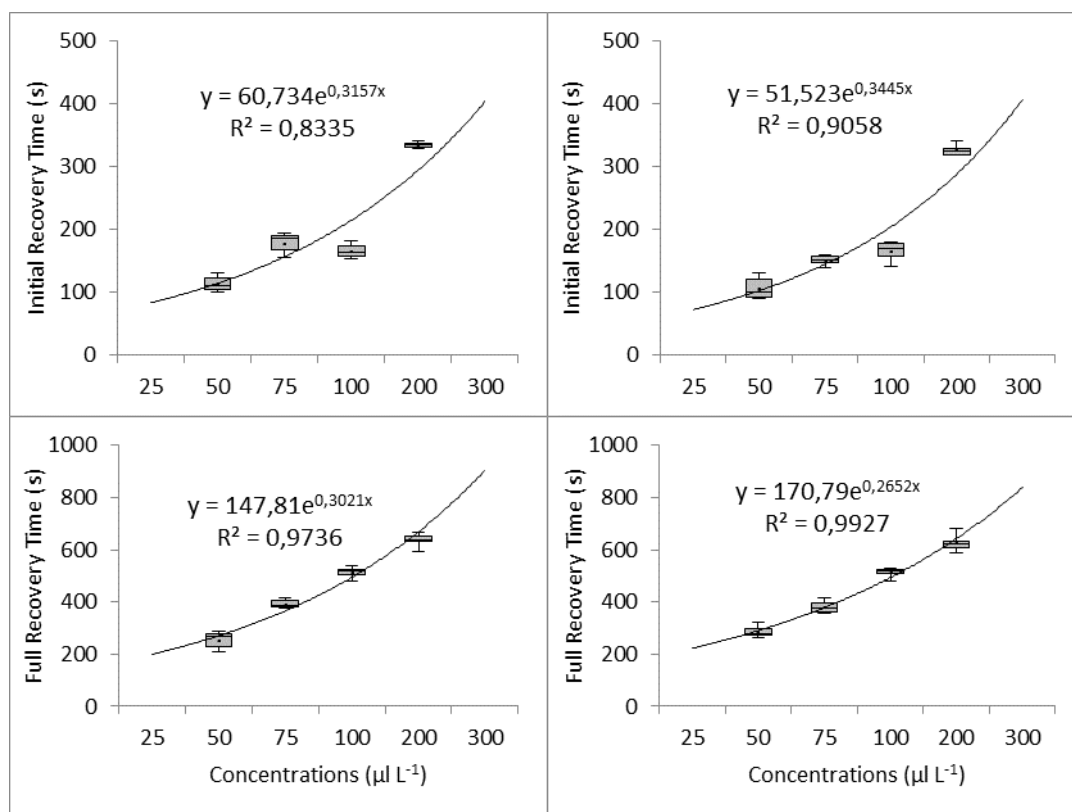


Fig. 1. Box plots of time (s) to induction (A_I and A_D) and recovery (R_I and R_F) distributions for *S. fryeri* and *L. caeruleus* anesthetized with various concentrations of CcEO. Relationships between induction/recovery stage times and essential oil concentrations for both fish species.

Induction and recovery times in both fish species were significantly concentration-dependant (Fig. 1). Negative relationships were recorded between A_D and R_F for CcEO (Fig. 2).

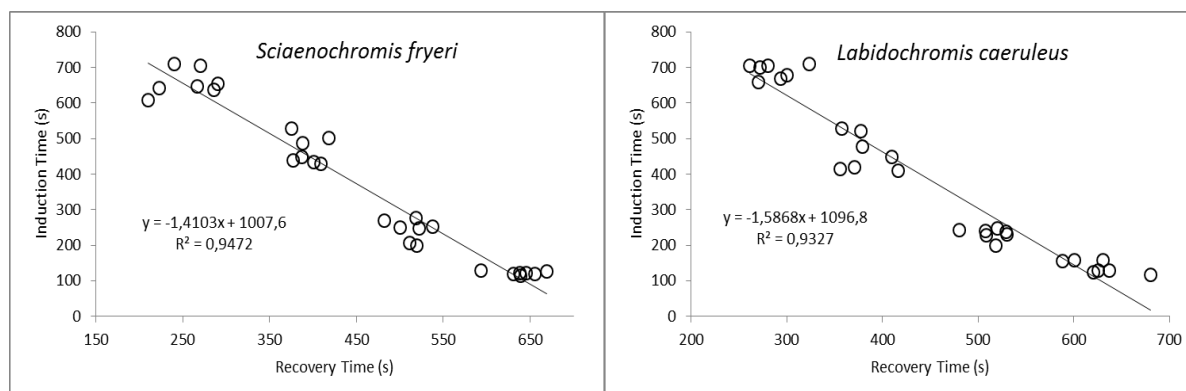


Fig. 2. Relationship between deep anesthesia (A_D) and full recovery (R_F) times in *L. caeruleus* and *S. fryeri* anesthetized by various concentrations CcEO.

Ethanol did not cause an anesthetic effect, abnormal behaviour, or mortality in treated fish. Fish started to feed almost 2 hours later (R_F). Neither mortality nor abnormal behaviour in fish was recorded 24 hours after trials.

Discussion

In the present study, *S. fryeri* and *L. caeruleus* were exposed to different concentrations of CcEO. Our findings showed that CcEO was an effective anesthetic agent. According to our knowledge, no other reports have been found on the anesthetics efficacy of CcEO on fish. The analgesic and anti-inflammatory activities of *C. citratus* infusion have been validated in rats (Garcia et al., 2015).

C. citratus (Family Poaceae) yields an essential oil with high content of citral (geranial and neral) (Viana et al., 2000; Paranagama et al., 2003). In the present study, the main components of CcEO were geranial (44.74 %), neral (31.00 %), geraniol (8.08 %) and geranyl acetate (5.98 %) (Table 1). *A. triphylla* and *C. flexuosus* essential oils with high α -citral (geranial) and β -citral (neral) contents induced deep anesthesia in *Rhamdia quelen* (Santos et al. 2017). These results showed that the citral components have anesthetic properties in fish species.

In aquaculture, handling invariably causes stress in fish and there are negative impacts of stress. These can be prevented or decreased by the use of anesthesia. Induction and recovery of fish within a certain time range is necessary. Research has indicated that A_D and R_F times should be no more than 3 min and 10 min, respectively (Mylonas et al., 2005; Perdikaris et al., 2010; Bodur et al., 2018). CcEO was found to be effective as anesthetic for *S. fryeri* and *L. caeruleus*. Increased concentrations lead to faster induction and prolonged recovery (Table 3, Fig. 1). Deep anesthesia can be achieved in *S. fryeri* and *L. caeruleus* at 200 μ L. This low concentration was optimal according to aforementioned criteria. Another natural and similar agent, *Cymbopogon flexuosus* has also been reported as an anesthetic agent for a number of fish species (Limma-Netto et al., 2016; Limma-Netto et al., 2017; Santos et al., 2017). In these publications, it was recommended that the concentrations of *C. flexuosus* at 300 μ L provided deep anesthesia for tambacu and silver catfish (Limma-Netto et al., 2016; Santos et al., 2017), and 600 μ L for Nile tilapia juveniles (Limma-Netto et al., 2017). Differences in effective concentrations of essential oil can be explained by species and age differences of fish.

The time taken for fish to recover from anesthesia is generally dependent on the concentration of the applied anesthetic (Weyl et al., 1996). In the present study, recovery times were positively correlated with anesthetic concentrations, and negatively with induction times (Fig. 2). Negative relationship between recovery and induction times were reported in many other studies (Weyl et al., 1996; Gökçek et al., 2017; Can et al., 2018; Kizak et al., 2018). However, Limma-Netto et al. (2016) stated that the recovery time in tambacu, exposed to *C. flexuosus*, decreased with increasing concentrations of the agent. Anesthetic recovery times vary according to the anesthetic agent, water temperature, and species of fish used (Mylonas et al., 2005; Limma-Netto et al., 2016).

Previous studies revealed that some herbal essential oils such as *Lippia sidoides* and *Ocimum basilicum*, could be inappropriate for fish, because of the side effects such as sudden jumping behavior, high loss of mucus, involuntary muscle contraction and mortality (Silva et al., 2013; Correia et al., 2018). It was also reported that *L. sidoides* essential oil with 300 μ L achieved anesthesia, but recovery time exceeded 30 min. In the present study, no side effects or mortality were observed for CcEO on both fish species even though full recovery stage at the highest concentration was almost 20 min more than required.

In conclusion, *Cymbopogon citratus* essential oil showed positive anesthetic properties on two ornamental fish species. Further studies are necessary to elucidate the anesthetic effects on other aquatic species as well as for different sizes of fish, and water quality conditions.

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